Cytokine 116 (2019) 70–77

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

SQSTM1/p62 regulates the production of IL-8 and MCP-1 in IL-1β-stimulated human retinal pigment epithelial cells

Eveliina Korhonen[a,*], Niina Piippo[a], Maria Hytti[a], Juha M.T. Hyttinen[b], Kai Kaarniranta[b,c], Anu Kauppinen[a]

[a] School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, P.O. Box 1627, FI-70211 Kuopio, Finland
[b] Department of Ophthalmology, Institute of Clinical Medicine, University of Eastern Finland, P.O. Box 1627, FI-70211 Kuopio, Finland
[c] Department of Ophthalmology, Kuopio University Hospital, P.O. Box 100, FI-70029 Kuopio, Finland

ARTICLE INFO

Keywords:
SQSTM1/p62
Interleukin-8
Monocyte chemoattractant protein-1
Inflammasome
Retinal pigment epithelium

ABSTRACT

Age-related macular degeneration (AMD) is a complex eye disease in which decline in autophagy leads to the accumulation of sequestosome 1/p62 (SQSTM1/p62)-labeled waste material inside the retinal pigment epithelial (RPE) cells, and the condition results in activation of the inflammasome signaling and IL-1β secretion. Here, we have studied the role of SQSTM1/p62 in the production of IL-6, IL-8, and MCP-1 in the presence or absence of IL-1β. SQSTM1/p62 was either overexpressed or silenced in ARPE-19 cells, which were then exposed to IL-1β. Alternatively, bafilomycin A was used to demonstrate the functional decline of autophagy with increased SQSTM1/p62 levels. The protein concentration of SQSTM1/p62 was measured using the western blot technique, and interleukin levels were determined by ELISA. In IL-1β-loaded RPE cells, SQSTM1/p62 depletion and overexpression increased the production of MCP-1 and IL-8, respectively. Neither knock-down nor overexpression of SQSTM1/p62 induced the release of IL-6. Our data suggest that SQSTM1/p62 is a significant factor in inflammatory responses, especially following the inflammasome activation.

1. Introduction

Age-related macular degeneration (AMD) is a degenerative eye disease, which is the leading cause of blindness among the elderly in Western countries. Due to increased life expectancies, it is predicted that the number of patients will increase in the future, reaching 288 million by 2040 [1]. The retinal pigment epithelium (RPE), a cell monolayer in the posterior part of the eye, plays a crucial role in the pathogenesis of AMD [2]. Its main functions include the absorption of light, the maintenance of the nutrient level of the photoreceptors, immune functions of the macula, as well as phagocytosis and degradation of photoreceptor outer segments (POS) [2]. It has been estimated that RPE cells engulf approximately 3 hundred million outer segment discs during the 70-years life span of a human being [3]. Therefore, the phagocytosis of POS material in a process called heterophagy, is a massive metabolic challenge for lysosomal pathways, which can become overburdened in aged RPE cells [2].

Macroautophagy, hereafter called as autophagy, is a regulated lysosomal degradation system responsible for the removal of cellular waste materials, such as aggregated proteins and dysfunctional cell organelles [4–5]. Autophagy is an adaptive process to reduce stress, promote the cell survival, and circulate nutrients [5]. SQSTM1/p62 is an autophagy substrate employed for binding and delivering ubiquitinated material prior to their decomposition in autophagosomes [6–8]. Upon localization into autophagosomes, SQSTM1/p62 interacts with microtubule-associated protein 1 light chain 3 (MAP1LC3, also known as LC3) via its LC3-interacting region (LIR) and becomes degraded along with the cargo of autophagosomes by the lysosomal enzymes [4,9]. We have previously shown that the decline in the intracellular clearance results in the accumulation of SQSTM1/p62 inside the RPE cells [10–11] as well as in the foveomacular area of AMD patients [10].

In addition to autophagosomes and autophagy substrates, SQSTM1/p62 can be located in both the cytoplasm and the nucleus [12]. Due to its several functional domains, SQSTM1/p62 is a scaffold protein for many signaling pathways [6,8,12]. For example, its domains regulate the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) by binding and thus inhibiting the function of kelch-like ECH-associated protein 1 (KEAP1) [13]. On the other hand, the transcription factor NFE2L2/Nrf2 can control the expression of SQSTM1/p62 [14]. Additionally, SQSTM1/p62 is also known to upregulate apoptosis, impaire adipogenesis [8,15] as well as activate the nuclear factor kappa B (NF-κB) via its TNF receptor associated factor 6 (TRAF6)-binding domain

* Corresponding author.
E-mail address: eveliina.korhonen@uef.fi (E. Korhonen).

https://doi.org/10.1016/j.cyto.2018.12.015
Received 10 September 2018; Received in revised form 19 December 2018; Accepted 20 December 2018
Available online 24 January 2019
1043-4666/

[16]. Moreover, SQSTM1/p62 has the ability to bind p38 and regulate cytokine-dependent p38 MAPK kinase activation, most likely via its N-terminal p38 interaction (NPI) and C-terminal p38 interaction (CPI) domains [17,18].

Together with malfunctional protein clearance, inflammation is a major factor in the pathogenesis of AMD [19]. We have previously shown that dysregulated intracellular protein clearance results in the activation of the NLR family pyrin domain-containing 3 (NLRP3) inflammasome in human RPE cells [20]. Our data also suggested that the inflammasome activation-induced release of IL-1β further promoted the release of IL-8 from RPE cells in an autocrinic manner [20]. Therefore, it appears possible that the secretion of IL-1β can amplify the inflammation in RPE cells with impaired intracellular clearance. Since RPE cells with dysfunctional proteasomal and autophagic systems have an abundance of SQSTM1/p62, we have now examined whether this protein is required for the IL-1β-driven release of pro-inflammatory cytokines in human RPE cells. Since dysregulated retinal para-inflammation and accumulation of inflammatory cells into the subretina are known to play a role in the pathogenesis of AMD [19,21], this study can help us to understand better the mechanisms underpinning AMD and to identify more specific treatment targets for this chronic eye disease.

2. Materials and methods

2.1. EGFP-SQSTM1/p62 fusion plasmid construct

The open reading frame (ORF) of human SQSTM1/p62 cDNA (NCBI gene bank no. NM_003900) was amplified from insert plasmid purchased from RZPD (Deutsches Ressourcenzentrum für Genomforschung, Berlin, Germany, cat.no IRAUp969A0698D). The ORF was amplified with a high-fidelity DNA polymerase (Phusion Hot start DNA polymerase, Finnzymes, Finland). The following primers were used: sense 5′-ATA *CTC GAG* at **ATG** GCG TCG CTC ACC; reverse 5′-TAT *AAG CTT* a **TCA** CAA CGG CGG GGG ATG. The added restriction digestion sites for *Xho*I and *Hin*dIII are shown in italics and the translation initiation and termination sites are in boldface. The additional bases enabling in-frame cloning are in minuscules.

The sticky ends for the amplified SQSTM/p62 ORF as well as for the multiple cloning site of the vector pEGFP-control (Clontech, Mountain View, CA, USA) were produced with restriction endonucleases *Xho*I and *Hin*dIII (MBI Fermentas, Vilnius, Lithuania). Ligated (T4 DNA Ligase, Roche, Basel, Switzerland) DNA, forming a fusion gene of EGFP and human SQSTM/p62, was transfected into competent DH5α *E. coli* cells, which were prepared using the protocol of Inoue and others [22]. The bacteria were cultured and the plasmids purified according to standard methods [23], and their integrity was determined initially by restriction endonuclease digestion analysis and finally by sequencing the two junction sites and neighbouring regions.

2.2. Cell stimulations

The human retinal pigment epithelial cell line (ARPE-19) was purchased from the American Type Culture Collection (ATCC). The cells were cultured in DMEM/F12 (1:1) growth medium (Life Technologies, Paisley, UK) containing 10% inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 2 mM L-glutamine (Lonza, Walkersville, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Lonza) at +37 °C in humidified conditions with 5% CO_2. Passages 26–33 were used in the present study.

In the transfection experiments, ARPE-19 cells were seeded onto 12-well plates at a density of 1×10^5 cells/ml and grown in serum-containing medium until 60–80% confluent. In the siRNA experiments, SQSTM1/p62 siRNA (cat.no s16962, 30 nM) or non-silencing siRNA (cat.no AM4611, 30 nM) was administered into the wells along with the siPORT Amine transfection reagent (all from Ambion, Austin, TX, USA) in serum-containing medium. In the SQSTM1/p62 overexpression experiments, EGFP-SQSTM1/p62 plasmid construct (1 μg) or control EGFP plasmid construct (1 μg) was added into the wells along with the ExGen500 transfection reagent (Thermo Fischer Scientific, Waltham, USA) in serum containing medium. After 24 h incubation, the wells were washed with serum-free medium and recombinant human IL-1β (10 pg/ml; Invitrogen, MD, USA) was delivered to cells in medium without serum. Thereafter, the cultures were incubated for 24 h at +37 °C in humidified conditions with 5% CO_2.

In the autophagy inhibition studies, ARPE-19 cells were seeded onto 12-well plates at a density of 2×10^5 cells/ml. Fully confluent cell layers were washed with serum-free medium, and the medium was replaced by fresh one. Cells were exposed to bafilomycin A (50 nM; Sigma) for 24 h with the subsequent treatment with recombinant human IL-1β exposure, as mentioned above. Subsequently, cell culture media were collected and centrifuged at 380 × *g* for 10 min. The supernatants were transferred into new tubes. The cells were washed once with ice-cold 1 × DPBS (Life Technologies) and lysed with M-PER solution according to the manufacturer's instructions (Thermo Scientific, Waltham, MA, USA). The cell lysates were centrifuged at 16 000 × *g* for 10 min at +4 °C and the supernatants were transferred into fresh tubes. All samples were stored at −20 °C until analysed.

2.3. Enzyme-linked immunosorbent assay (ELISA)

IL-8, IL-6 (BD Biosciences, San Diego, CA, USA), and MCP-1 (eBioscience, San Diego, CA, USA) were measured using the ELISA method from cell culture medium samples according to the manufacturers' instructions. Absorbance values were measured at 450 nm with the reference wavelength of 655 nm using a spectrophotometer (Biorad, Model 550, Hercules, CA, USA).

2.4. Western blot

Protein concentration was measured using the Bradford method, and equal amounts of protein (11–25 μg) in the cell lysates were run in 15% SDS-PAGE gels and wet-blotted overnight onto nitrocellulose membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Ponceau S (Sigma-Aldrich, St. Louis, MO, USA) staining was used to ensure the accurate protein transfer from the gels to the membranes. The membranes were blocked with 3% fat-free milk in 0.3% Tween20/PBS for 2 h at room temperature (RT). Thereafter, the membranes were incubated with mouse monoclonal antibodies for SQSTM1/p62 (1:1000 in 0.5% BSA in 0.3% T-PBS; Santa Cruz Biotechnology, Inc. Dallas, TX, USA) overnight at +4 °C, GAPDH (1:15000 in 0.1% T-PBS; Abcam, Cambridge, UK) for 2 h at RT, or with alpha-tubulin (1:8000 in 1% milk in 0.05% Tween/PBS; Sigma) for 15 min at RT. The membranes were washed 3 × 5 min with the respective washing buffer followed by 2 h incubation for SQSTM1/p62, 1 h incubation for GAPDH, or 15 min incubation for alpha-tubulin at RT with a horse-radish peroxidase (HRP)-conjugated anti-mouse IgG antibody (1:10000 in 0.3% T-PBS for SQSTM1/p62, 1:12 000 in 0.1% T-PBS for GAPDH, and 1:10 000 in 1% milk in 0.05% T-PBS for alpha-tubulin, GE Healthcare). The washing steps were repeated and SQSTM1/p62, GAPDH, or alpha-tubulin protein-antibody complexes were detected using the enhanced chemiluminescent assay after the substrate (Millipore, Billerica, MA, USA) addition. All results were quantified using the ImageJ program (http://rsb.info.nih.gov/ij/index.html).

2.5. Statistical analyses

All data were analyzed using the GraphPad Prism program (GraphPad Software, San Diego, CA, USA, version 7.04). Pairwise comparisons were performed using the Mann-Whitney *U*-test, and *p*-values 0.05 or less were considered as statistically significant.

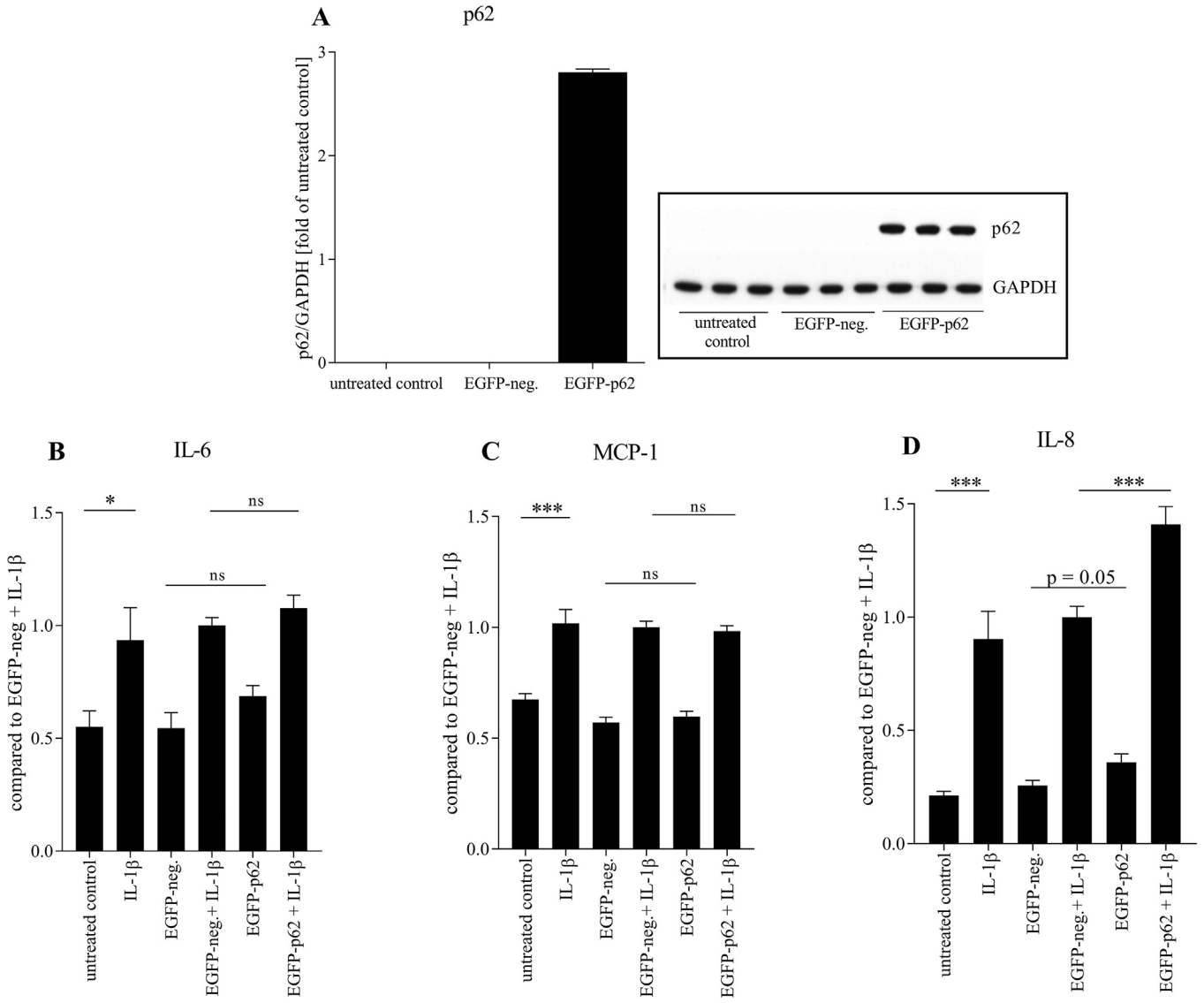

Fig. 1. The effect of SQSTM1/p62-overexpression on ARPE-19 cells. (A) Levels of SQSTM1/p62 were detected by western blot and normalized to the internal control protein, GAPDH. (B) The levels of secreted IL-6, (C) MCP-1, and (D) IL-8 were determined from ARPE-19 cells, which were initially transfected with pEGFP-SQSTM1/p62 or pEGFP constructs for 24 h followed by treatment with IL-1β for the next 24 h. Results (B–D) have been combined from three independent experiments with four parallel samples per group and are presented as a mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$, ns = not significant difference, Mann-Whitney *U*-test. EGFP-neg. = EGFP-control.

3. Results

3.1. SQSTM1/p62 overexpression triggers modest secretion of IL-8

ARPE-19 cells were treated with the pEGFP-SQSTM1/p62 or control pEGFP constructs, and the success of transfections was monitored by measuring the SQSTM1/p62 protein levels with western blot technology. As expected, the EGFP-SQSTM1/p62 overexpression increased the cellular SQSTM1/p62 levels so that they were 15.8 times higher when compared to the EGFP control or untreated control cells (Fig. 1A). Cells overexpressing EGFP-SQSTM1/p62 only slightly increased the secretion of IL-6, and the difference was not statistically significant when compared to cells treated with pEGFP alone (Fig. 1B; 6.3 pg/ml vs. 5.2 pg/ml, respectively; $p = 0.1830$, Mann-Whitney *U*-test). Furthermore, the levels of MCP-1 were not increased after the EGFP-SQSTM1/p62 overexpression (Fig. 1C; mean in EGFP-SQSTM1/p62: 658.2 pg/ml and EGFP-control: 638.9 pg/ml, $p = 0.5899$). The EGFP control merely decreased the secretion of MCP-1 when compared to untreated cells (Fig. 1C; 638.9 pg/ml vs. 736.9 pg/ml, respectively; $p = 0.0145$). This was at least partly due to the transfection reagent, which alone decreased the release of MCP-1 as compared to untreated control cells (Supplementary Fig. 1A; $p = 0.0207$). In contrast to IL-6 or MCP-1, EGFP-SQSTM1/p62 overexpression increased the levels of IL-8 when compared to cells treated with pEGFP alone (Fig. 1D; EGFP-SQSTM1/p62: 193.8 pg/ml vs. EGFP-control: 138.0 pg/ml, $p = 0.0519$). Overexpression of SQSTM1/p62 increased the levels of IL-8 ca. 40% when compared to the transfection control.

3.2. Overexpression of SQSTM1/p62 increases the levels of IL-8 in IL-1β-stimulated RPE cells

We have previously shown that 1 pg IL-1β can induce the secretion of IL-8 in ARPE-19 cells [20]. In the present study, RPE cells were treated with 10 pg IL-1β, which was sufficient to induce the release of IL-6 (mean in untreated control: 5.29 pg/ml and IL-1β: 8.84 pg/ml, $p = 0.0449$; Fig. 1B). Exposure to IL-1β also induced the secretion of MCP-1 (mean in untreated control: 736.9 pg/ml and IL-1β: 1193.7 pg/ml, $p = 0.0005$; Fig. 1C) and IL-8 (mean in untreated control: 114.5 pg/

ml and IL-1β: 496.2 pg/ml, $p < 0.0001$; Fig. 1D).

The presence of excessive amounts of SQSTM1/p62 in ARPE-19 cells during the IL-1β treatment did not affect the secretion of IL-6 ($p = 0.3474$) or MCP-1 ($p = 0.5512$) when compared to IL-1β-stimulated EGFP-control (means in IL-6: EGFP-SQSTM1/p62 + IL-1β: 9.96 pg/ml and EGFP + IL-1β: 9.59 pg/ml, Fig. 1B; means in MCP-1: EGFP-SQSTM1/p62 + IL-1β: 1102.5 pg/ml and EGFP + IL-1β: 1140.5 pg/ml, Fig. 1C). Unlike IL-6 or MCP-1, the secretion of IL-8 was increased upon the IL-1β treatment in the presence of SQSTM1/p62 (Fig. 1D). SQSTM1/p62 overexpression-induced secretion of IL-8 was ca. 60% higher when compared to cells exposed to IL-1β alone (mean in EGFP-SQSTM1/p62 + IL-1β: 786.4 pg/ml and IL-1β: 496.2 pg/ml, $p = 0.0145$; Fig. 1D) and ca. 40% higher when compared to EGFP + IL-1β control (mean in EGFP- SQSTM1/p62 + IL-1β: 786.4 pg/ml and EGFP + IL-1β: 561.6 pg/ml, $p = 0.0002$; Fig. 1D). The concentration range of IL-8 was 496 – 786 pg/ml and 115–194 pg/ml with and without IL-1β treatment, respectively.

3.3. Silencing of SQSTM1/p62 increases the secretion of IL-8 and MCP-1

Next, we studied whether the absence of SQSTM1/p62 exerted any effect on cytokine and chemokine production, with the expression of SQSTM1/p62 after the specific small interfering RNA (siRNA) treatment being detected by western blot. SQSTM1/p62-siRNA entirely blocked the expression of SQSTM1/p62 in ARPE-19 cells when compared to cells treated with non-specific siRNA or left untreated (Fig. 2A). Neither transfection itself nor depletion of SQSTM1/p62 affected the levels of IL-6 (Fig. 2B; SQSTM1/p62 siRNA: 4.7 pg/ml and non-silencing siRNA: 4.2 pg/ml $p = 0.1402$). Instead, SQSTM1/p62 siRNA increased the release of MCP-1 when compared to cells exposed to non-specific siRNA (Fig. 2C; means 987.371 pg/ml and 709.514 pg/ml, respectively; $p < 0.0001$). In addition, SQSTM1/p62 siRNA statistically significantly increased the secretion of IL-8 in comparison to cells exposed to a non-specific siRNA (Fig. 2D; means 321.5 pg/ml vs. 288.3 pg/ml, respectively; $p = 0.0394$) but the increase was only ca. 10%. Also the non-specific siRNA yielded significantly higher levels of IL-8 when compared to untreated control cells. This was mainly due to effect of transfection reagent (Supplementary Fig. 1B; $p = 0.0022$).

3.4. Absence of SQSTM1/p62 does not affect the IL-8 levels but increases the release of MCP-1 from IL-β-stimulated RPE cells

The levels of IL-8, IL-6, and MCP-1 were measured from SQSTM1/p62-depleted cells exposed to IL-1β. ARPE-19 cells did not secrete significantly greater amounts of IL-6 ($p = 0.1088$) if the cells were treated with SQSTM1/p62 siRNA + IL-1β than those exposed to non-specific siRNA + IL-1β or IL-1β alone (mean in SQSTM1/p62 siRNA + IL-1β: 5.67 pg/ml, non-specific siRNA + IL-1β: 6.48 pg/ml, and IL-1β: 4.83 pg/ml, Fig. 2B). Instead, the loss of SQSTM1/p62 in the IL-1β-stimulated cells induced the secretion of MCP-1 (mean in SQSTM1/p62 siRNA + IL-1β: 1068.04 pg/ml and non-specific siRNA + IL-1β: 901.61 pg/ml, $p = 0.0295$; Fig. 2C), which is in line with the experiments without IL-1β. Although non-specific siRNA together with IL-1β treatment increased the IL-8 levels when compared to IL-1β-control cells with normal SQSTM1/p62 levels ($p < 0.0001$), the difference between SQSTM1/p62 siRNA + IL-1β and non-specific siRNA + IL-1β samples was not statistically significant ($p = 0.4569$, mean in SQSTM1/p62 siRNA + IL-1β: 385.12 pg/ml, non-specific siRNA + IL-1β: 399.26 pg/ml, and IL-1β: 262.35 pg/ml; Fig. 2D). This indicates that the depletion of SQSTM1/p62 did not specifically affect the secretion of IL-8 in IL-1β-treated cells.

3.5. Blockade of autophagy flux decreases the secretion of IL-6 and MCP-1 but increases the levels of IL-8

ARPE-19 cells were lastly treated by an inhibitor of autophagosomal H^+-ATPase, bafilomycin A (hereafter referred to as BafA), which increased the levels of SQSTM1/p62 both in the absence and presence of IL-1β (Fig. 3A). The levels of IL-6 were reduced by 6.4 and 3.8 times upon the BafA or BafA + IL-1β exposures when compared to untreated control ($p < 0.0001$) or IL-1β-treated control cells ($p < 0.0001$), respectively (mean in untreated control: 58.6 pg/ml, BafA: 9.2 pg/ml, IL-1β: 75.0 pg/ml, and BafA + IL-1β: 15.01 pg/ml; Fig. 3B). In addition, the levels of MCP-1 were decreased by 2.1 and 1.7 times after the BafA and BafA + IL-1β treatments when compared to the untreated control ($p < 0.0001$) or IL-1β-stimulated control cells ($p < 0.0001$), respectively (mean in untreated control: 1544.7 pg/ml, BafA: 717.6 pg/ml, IL-1β: 2378.2 pg/ml, and BafA + IL-1β: 846.0 pg/ml; Fig. 3C). The results of IL-8 were in line with the data obtained in the SQSTM1/p62 overexpression experiments (Fig. 1) since BafA treatment without and with IL-1β increased the secretion of IL-8 4.6 and 1.7 times when compared to untreated ($p < 0.0001$) and IL-1β-treated cells ($p = 0.0009$), respectively (mean in untreated control: 116.3 pg/ml, BafA: 529.5 pg/ml, IL-1β: 499.7 pg/ml, and BafA + IL-1β: 835.5 pg/ml; Fig. 3D).

4. Discussion

The increased intracellular accumulation of SQSTM1/p62 in the foveomacular area of AMD patients is evidenced for a dysfunctional protein clearance in the pathogenesis of AMD [10]. We have recently reported that the impaired protein clearance promotes the NLRP3 inflammasome-mediated IL-1β production in human RPE cells, which further induces the release of IL-8 chemokine [20]. So far, the role of SQSTM1/p62 in modulating the production of cytokines and chemokines in RPE cells has remained unknown.

Our present experiments without IL-1β stimulation revealed that both the overexpression and silencing of SQSTM1/p62 induced the secretion of IL-8 in ARPE-19 cells. However, the lack of SQSTM1/p62 increased IL-8 only ca. 10%, whereas the effect of the overexpression of SQSTM1/p62 was ca. 40%. Our data with bafilomycin A (hereafter referred to as BafA) supported this finding since it increased the intracellular levels of SQSTM1/p62 and triggered the IL-8 secretion similarly to SQSTM1/p62-overexpressed RPE cells. The inhibition of autophagy by BafA has already previously been shown to increase the expression of upstream proteins involved in autophagy, including SQSTM1/p62, in RPE cells [10,11]. BafA-triggered IL-8 release is in line with the results of Yang et al. who revealed that chloroquine, another autophagy blocker, induced NF-κB activation-mediated expression of IL-8 mRNA in melanoma cells and squamous cell carcinoma cells [24]. Additionally, Yang et al. showed that BafA at concentrations of 10 to 50 nM induced IL-8 mRNA expression [24]. In the present study, 50 nM BafA was able to induce an elevated release of IL-8 chemokine from RPE cells.

The levels of IL-6 remained unchanged upon different amounts of SQSTM1/p62 but the blockade of SQSTM1/p62 increased the release of MCP-1, suggesting that some SQSTM1/p62 is needed for maintaining the expression of MCP-1 at the basal level. Interestingly, although BafA treatment with excessive amounts of SQSTM1/p62 increased the release of IL-8, the productions of IL-6 and MCP-1 were reduced. SQSTM1/p62 has shown its anti-inflammatory properties by regulating myeloid differentiation primary response 88 (MyD88)-dependent expression of IL-6 [25]. Since our interleukin-1 receptor (IL-1R)-mediated stimulation differed from the signaling pathway of Toll-like receptors (TLRs), MyD88 may not be the only target for the anti-inflammatory effects of SQSTM1/p62. Kim and Ozato also found that excessive amounts of SQSTM1/p62 prevented the secretion of IL-6, whereas the loss of SQSTM1/p62 increased the release of IL-6 in macrophages [26]. The absence of SQSTM1/p62 has been reported to reduce the secretion IL-6 in human keratinocytes [27], implying that cell type plays a role in determining the response. In the present study, we did not find any increased or decreased IL-6 levels following the overexpression or the silencing of SQSTM1/p62. Therefore, the reduced IL-6 levels only in

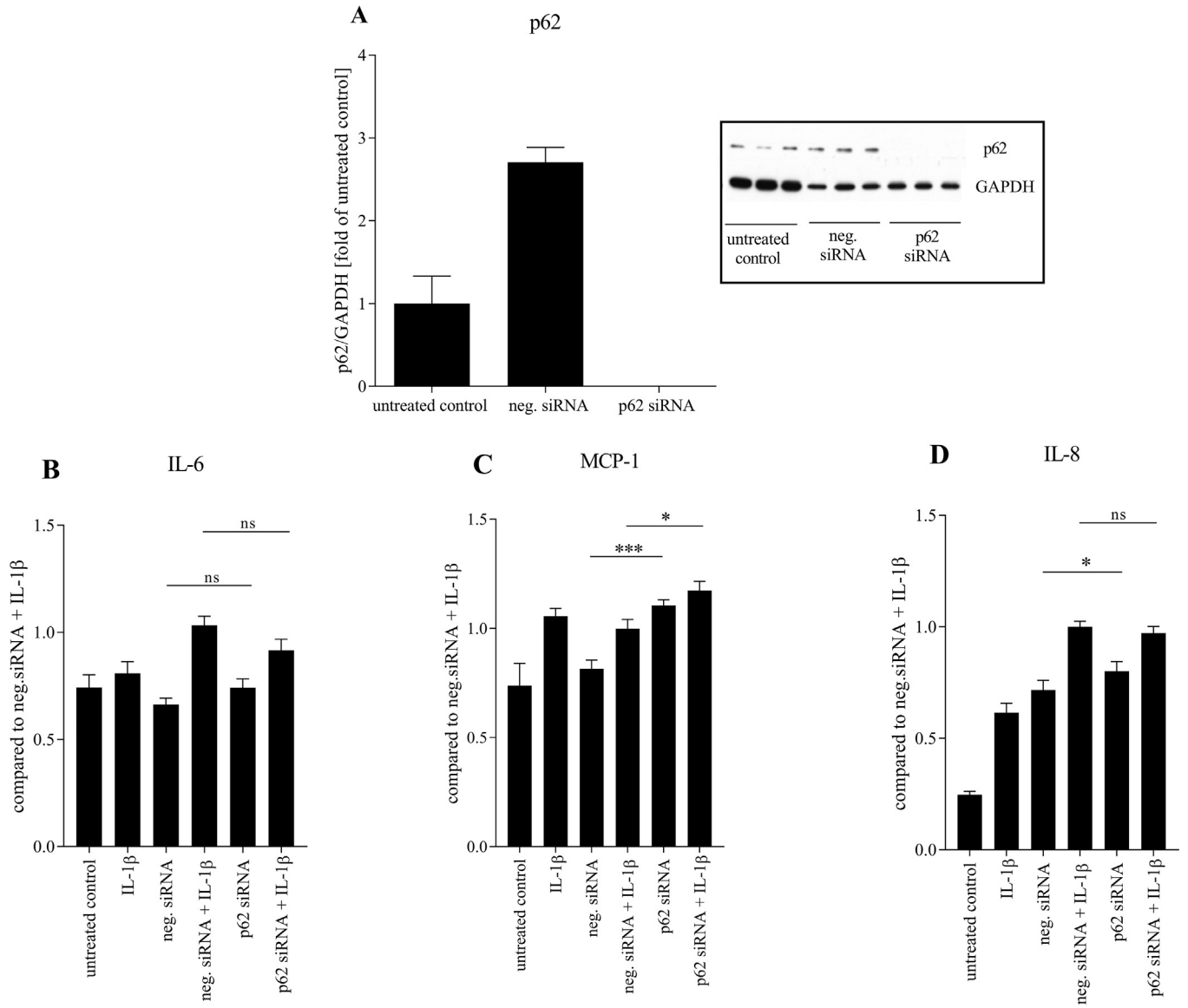

Fig. 2. The effect SQSTM1/p62 knockdown on ARPE-19 cells. (A) Protein content of SQSTM1/p62 was measured by western blot and the result of SQSTM1/p62 was normalized to the internal control, GAPDH. (B) The levels of secreted IL-6, (C) MCP-1, and D) IL-8 were measured using the ELISA method from ARPE-19 cells, which were initially transfected by SQSTM1/p62 siRNA or non-specific siRNA for 24 h followed by treatment with IL-1β for the next 24 h. Results (B–D) have been combined from three independent experiments with six (B and D) or three to six (C) parallel samples per group and are shown as a mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$, ns = not significant difference, Mann-Whitney *U*-test. Neg. siRNA = non-specific siRNA.

BafA-treated ARPE-19 cells may have occurred via the pH-dependent mechanism similarly to chloroquine-stimulated human monocyte cell cultures [28].

When ARPE-19 cells overexpressing or lacking of SQSTM1/p62 were stimulated with IL-1β, we found that only excessive amounts of SQSTM1/p62 increased the release of IL-8. This result is in line with those of Kawai and others who reported that the loss of SQSTM1/p62 in IL-1β-stimulated HeLa cells downregulated the phosphorylation of p38 and destabilized IL-8 mRNA, suggesting that reduced levels of SQSTM1/p62 are able to protect cells from inflammation [17]. Accordingly, we found that the loss of SQSTM1/p62 along with IL-1β had no effect on the release of IL-8, suggesting the stable levels of IL-8 after IL-1β stimulation.

The depleted but not overexpressed SQSTM1/p62 increased the levels of MCP-1 in IL-1β-stimulated cells, which is in line with the finding without IL-1β indicating that a certain amount of SQSTM1/p62 is required for maintaining cellular homeostasis and keeping MCP-1 at the basal level. Previously, it has been reported that innate defense regulator-1 (IDR-1) peptide can elevate MCP-1 levels in human peripheral blood mononuclear cells (PBMCs) [29] and thereafter, it has been shown that IDR-1 binds to the ZZ-type zinc finger (ZZ)-domain of SQSTM1/p62 [30]. One could hypothesize that in the absence of SQSTM1/p62, there is more unbound IDR-1 available in the cytoplasm to trigger the release of MCP-1. As SQSTM1/p62 has scaffold proteins for different cellular pathways [6,8], distinct results of IL-8, IL-6, and MCP-1 may be explained by the participation of the different domains of SQSTM1/p62. Although it has been known that SQSTM1/p62 can mediate both anti- and pro-inflammatory effects on cells [13,16], this study highlights that SQSTM1/p62 has a pivotal role in a regulation of inflammatory markers also in RPE cells.

In summary, the impaired protein clearance leads to the accumulation of SQSTM1/p62-labeled waste material inside the RPE cells [10,11], and these conditions cause the activation of the inflammasome in conjunction with IL-1β secretion [20]. According to the present study, SQSTM1/p62 contributes to increased secretion of IL-8 upon IL-1β stimulation in human RPE cells (Fig. 4). Some SQSTM1/p62 is

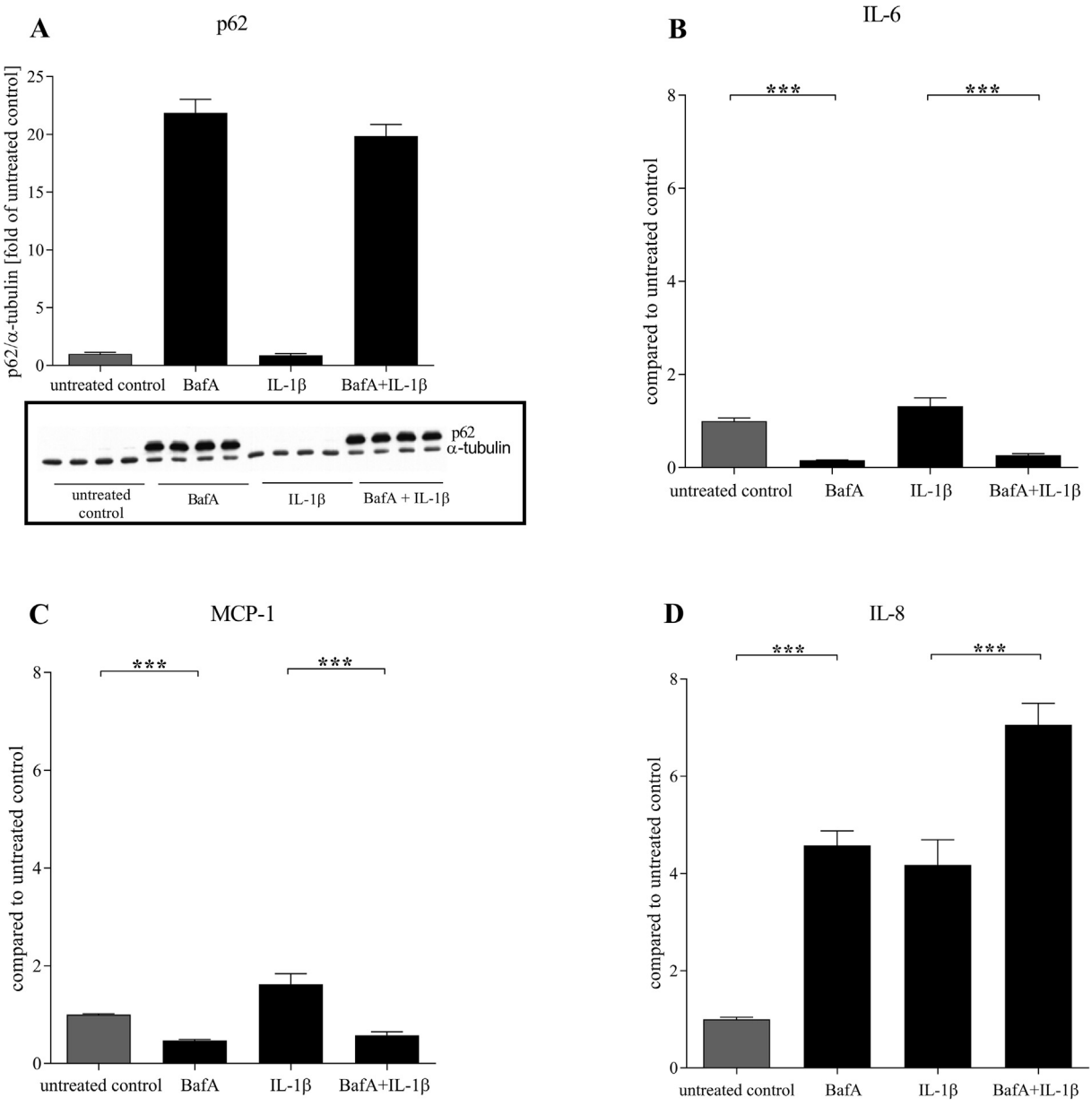

Fig. 3. The effect of bafilomycin A on SQSTM1/p62 and cytokine levels in the absence or presence of IL-1β in ARPE-19 cells. (A) The protein content of SQSTM1/p62 was measured by western blot and the results were normalized to the internal control, alpha-tubulin. (B) Secretions of IL-6, (C) MCP-1, and (D) IL-8 were measured by ELISA from RPE cells stimulated with bafilomycin A for 24 h followed by an exposure with IL-1β for the next 24 h. Results (B–D) have been combined from two independent experiments with six (B–C) or five to six (D) parallel samples and are shown as a mean ± SEM. $^{***}p < 0.001$, Mann-Whitney *U*-test.

needed also for functional autophagy and cellular homeostasis [6]. Therefore, the avoidance of excessive SQSTM1/p62 levels might help to regulate the secretion of IL-8 without increasing the production of another chemokine, MCP-1. Since IL-8 and MCP-1 are chemokines that recruit neutrophils and monocytes to an inflamed tissue, respectively [31,32], the regulation of SQSTM1/p62 as well as the control of the IL-1β secretion could help to prevent chronic inflammation in the retina. Neutrophils further attract monocytes into the site of inflammation [33], which highlights the importance of IL-8 regulation in the prevention of local inflammation. Currently, there is no proper approved treatment available for AMD patients who are suffering from the dry form of the disease; these make up 80% of all AMD cases [34]. As inflammation is involved in AMD pathogenesis [19], our present study provides additional information to be considered when seeking new therapeutic approaches.

Acknowledgements

We warmly acknowledge Dr. Ewen MacDonald for the language revision, and Res. Dir. Emeritus Antero Salminen for his valuable collaboration, discussions, and critical review of the manuscript. This study was financially supported by Finnish Cultural Foundation (Central and North-Savo Regional Fund), Emil Aaltonen Foundation, the Academy of Finland (Health Research Council projects AK297267 and AK307341), Sokeain ystävät ry, and Silmä- ja kudospankkisäätiö.

Author contributions

A.K. and E.K. conceived and designed the study; E.K., N.P., and M.H. performed the experiments; E.K. analyzed the data; J.M.T.H. designed and prepared the EGFP-SQSTM1/p62 fusion plasmid construct; A.K. and K.K. contributed to materials and analysis tools; E.K. and A.K.

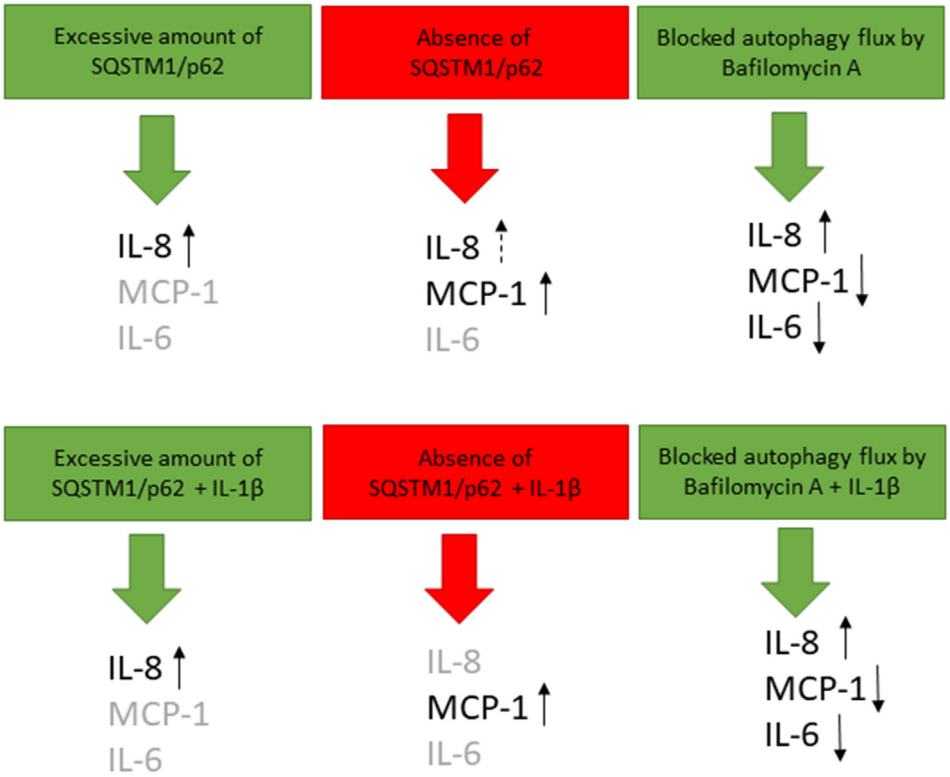

Fig. 4. Summary picture of the main results.

wrote the paper, and others critically reviewed, commented, and provided suggestions to the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2018.12.015.

References

[1] W.L. Wong, X. Su, X. Li, C.M.G. Cheung, R. Klein, C. Cheng, T.Y. Wong, Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis, Lancet Glob Health 2 (2) (2014) 106.
[2] K. Kaarniranta, D. Sinha, J. Blasiak, A. Kauppinen, Z. Veréb, A. Salminen, M.E. Boulton, G. Petrovski, Autophagy and heterophagy dysregulation leads to retinal pigment epithelium dysfunction and development of age-related macular degeneration, Autophagy 9 (7) (2013) 973–984.
[3] J. Marshall, The ageing retina: physiology or pathology, Eye (Lond) 1 (Pt 2) (1987) 282–295.
[4] T. Lamark, T. Johansen, Aggrephagy: selective disposal of protein aggregates by macroautophagy, Int. J. Cell Biol. 2012 (2012) 736905.
[5] Z. Yang, D.J. Klionsky, Eaten alive: a history of macroautophagy, Nat. Cell Biol. 12 (9) (2010) 814–822.
[6] X. Lin, S. Li, Y. Zhao, X. Ma, K. Zhang, X. He, Z. Wang, Interaction domains of p62: a bridge between p62 and selective autophagy, DNA Cell Biol. 32 (5) (2013) 220–227.
[7] W.J. Liu, L. Ye, W.F. Huang, L.J. Guo, Z.G. Xu, H.L. Wu, C. Yang, H.F. Liu, p62 links the autophagy pathway and the ubiqutin-proteasome system upon ubiquitinated protein degradation, Cell Mol. Biol. Lett. 21 (2016) 29.
[8] M. Komatsu, S. Kageyama, Y. Ichimura, p62/SQSTM1/A170: physiology and pathology, Pharmacol. Res. 66 (6) (2012) 457–462.
[9] S. Pankiv, T.H. Clausen, T. Lamark, et al., p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy, J. Biol. Chem. 282 (33) (2007) 24131–24145.
[10] J. Viiri, M. Amadio, N. Marchesi, et al., Autophagy activation clears ELAVL1/HuR-mediated accumulation of SQSTM1/p62 during proteasomal inhibition in human retinal pigment epithelial cells, PloS One 8 (7) (2013) e69563.
[11] J. Viiri, J.M.T. Hyttinen, T. Ryhänen, et al., p62/sequestosome 1 as a regulator of proteasome inhibitor-induced autophagy in human retinal pigment epithelial cells, Mol. Vis. 16 (2010) 1399–1414.
[12] Y. Katsuragi, Y. Ichimura, M. Komatsu, p62/SQSTM1 functions as a signaling hub and an autophagy adaptor, FEBS J. 282 (24) (2015) 4672–4678.
[13] M. Komatsu, H. Kurokawa, S. Waguri, et al., The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1, Nat. Cell Biol. 12 (3) (2010) 213–223.
[14] I. Johansson, V.T. Monsen, K. Pettersen, J. Mildenberger, K. Misund, K. Kaarniranta, S. Schønberg, G. Bjørkøy, The marine n-3 PUFA DHA evokes cytoprotection against oxidative stress and protein misfolding by inducing autophagy and NFE2L2 in human retinal pigment epithelial cells, Autophagy 11 (9) (2015) 1636–1651.
[15] Z. Jin, Y. Li, R. Pitti, D. Lawrence, V.C. Pham, J.R. Lill, A. Ashkenazi, Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling, Cell 137 (4) (2009) 721–735.
[16] J. Moscat, M.T. Diaz-Meco, M.W. Wooten, Signal integration and diversification through the p62 scaffold protein, Trends Biochem. Sci. 32 (2) (2007) 95–100.
[17] K. Kawai, A. Saito, T. Sudo, H. Osada, Specific regulation of cytokine-dependent p38 MAP kinase activation by p62/SQSTM1, J. Biochem. 143 (6) (2008) 765–772.
[18] T. Sudo, M. Maruyama, H. Osada, p62 functions as a p38 MAP kinase regulator, Biochem. Biophys. Res. Commun. 269 (2) (2000) 521–525.
[19] A. Kauppinen, J.J. Paterno, J. Blasiak, A. Salminen, K. Kaarniranta, Inflammation and its role in age-related macular degeneration, Cell Mol. Life Sci. 73 (9) (2016) 1765–1786.
[20] N. Piippo, A. Korkmaz, M. Hytti, K. Kinnunen, A. Salminen, M. Atalay, Kaarniranta K & Kauppinen A (2014): Decline in cellular clearance systems induces inflammasome signaling in human ARPE-19 cells, Biochim. Biophys. Acta 12 (1843) 3038–3046.
[21] M. Chen, H. Xu, Parainflammation, chronic inflammation, and age-related macular degeneration, J. Leukocyte Biol. 98 (5) (2015) 713–725.
[22] H. Inoue, H. Nojima, H. Okayama, High efficiency transformation of Escherichia coli with plasmids, Gene 96 (1) (1990) 23–28.
[23] J. Sambrook, E.F. Fritsch, T. Maniatis, Extraction and purification of plasmid DNA, second ed. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, NY, USA, 1990, pp. 1.25–1.28.
[24] S. Yang, L. Qiang, A. Sample, P. Shah, Y. He, NF-κB signaling activation induced by chloroquine requires autophagosome, p62 protein, and c-Jun N-terminal kinase (JNK) signaling and promotes tumor cell resistance, J. Biol. Chem. 292 (8) (2017) 3379–3388.
[25] T. Into, M. Inomata, S. Niida, Y. Murakami, K. Shibata, Regulation of MyD88 aggregation and the MyD88-dependent signaling pathway by sequestosome 1 and histone deacetylase 6, J. Biol. Chem. 285 (46) (2010) 35759–35769.
[26] J.Y. Kim, K. Ozato, The sequestosome 1/p62 attenuates cytokine gene expression in activated macrophages by inhibiting IFN regulatory factor 8 and TNF receptor-associated factor 6/NF-kappaB activity, J. Immunol. 182 (4) (2009) 2131–2140.
[27] H. Lee, D. Shin, J. Yuk, et al., Autophagy negatively regulates keratinocyte

inflammatory responses via scaffolding protein p62/SQSTM1, J. Immunol. 186 (2) (2011) 1248–1258.
[28] C. Jang, J. Choi, M. Byun, D. Jue, Chloroquine inhibits production of TNF-alpha, IL-1beta and IL-6 from lipopolysaccharide-stimulated human monocytes/macrophages by different modes, Rheumatology (Oxford) 45 (6) (2006) 703–710.
[29] M.G. Scott, E. Dullaghan, N. Mookherjee, et al., An anti-infective peptide that selectively modulates the innate immune response, Nat. Biotechnol. 25 (4) (2007) 465–472.
[30] H.B. Yu, A. Kielczewska, A. Rozek, et al., Sequestosome-1/p62 is the key intracellular target of innate defense regulator peptide, J. Biol. Chem. 284 (52) (2009) 36007–36011.
[31] M. Baggiolini, P. Loetscher, B. Moser, Interleukin-8 and the chemokine family, Int. J. Immunopharmacol. 17 (2) (1995) 103–108.
[32] S.L. Deshmane, S. Kremlev, S. Amini, B.E. Sawaya, Monocyte chemoattractant protein-1 (MCP-1): an overview, J. Interferon Cytokine Res. 29 (6) (2009) 313–326.
[33] F.K. Swirski, C.S. Robbins, Neutrophils usher monocytes into sites of inflammation, Circ. Res. 112 (5) (2013) 744–745.
[34] R. Velez-Montoya, S.C.N. Oliver, J.L. Olson, S.L. Fine, H. Quiroz-Mercado, N. Mandava, Current knowledge and trends in age-related macular degeneration: genetics, epidemiology, and prevention, Retina (Philadelphia, Pa.) 34 (3) (2014) 423–441.